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(54) Title: DNA SEQUENCE ENCODING PLANT 2-ACYLTRANSFERASE

(57) Abstract

Plants, particularly transgenic plants, may be produced having a 2-acyltransferase enzyme from *Limnanthes* with an altered substrate specificity compared to the native enzyme. For example, oil seed rape *Brassica napus* may contain the 2-acyltransferase transgene derived from *Limnanthes douglasii* in order to produce trierucin. The cDNA sequence of *Limnanthes douglasii* 2-acyltransferase and its equivalent protein sequence are disclosed.

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DNA SEQUENCE ENCODING PLANT 2-ACYLTRANSFERASE

This invention relates to modified plants. In particular, the invention relates to plants modified such that at least part of the plant (for example seeds of the plant) is capable of yielding a commercially useful oil.

Plants have long been a commercially valuable source of oil. Nutritional uses of plant-derived oils have hitherto been dominant, but attention is now turning additionally to plants as a source of industrially useful oils, for example as replacements for or improvements on mineral oils. Oil seeds, such as from rape, have a variety of lipids in them (Hildish & Williams, "Chemical Composition of Natural Lipids", Chapman Hall, London, 1964). There is considerable interest in altering lipid composition by the use of recombinant DNA technology (e.g. Knauf, *TIBtech*, February 1987, 40-47), but by no means all of the goals have been realised to date for a variety of reasons, in spite of the ever-increasing sophistication of the technology.

Success in tailoring the lipid content of plant-derived oils requires a firm understanding of the biochemistry and genes involved. Broadly, two approaches are available. First, plants may be modified to permit the synthesis of fatty acids which are new (for the plant); so, for example, laurate and/or stearate may be synthesised in rape. Secondly, the pattern and/or extent of incorporation of fatty acids into the glycerol backbone of the lipid may be altered. It is with this latter approach that the present invention is concerned.

Lipids are formed in plants by the addition of fatty acid

moieties onto the glycerol backbone by a series of acyl transferase enzymes. There are three positions on the glycerol molecule at which fatty acid (acyl) moieties may be substituted, and the substitution reached at each position is catalysed by a position-specific enzyme: the enzymes are glycerol-sn-3-phosphate acyltransferase (1-acyltransferase), 1-acyl-sn-glycerol-3-phosphate acyltransferase (2-acyltransferase) and sn-1,2-diacylglycerol acyltransferase (3-acyltransferase).

One, but not the only, current aim of "lipid engineering" in plants is to provide oils including lipids with a higher content of erucic (22:1) acid and/or oils containing trierucin. Erucic acid-containing lipids are commercially desirable for a number of purposes, particularly as replacements to or supplements for mineral oils in certain circumstances, as alluded to above. In the case of oil seed rape (*Brassica napus*), one of the most significant oil producing crops in cultivation today, the specificity of the 2-acyltransferase enzyme positively discriminates against the incorporation of erucic acid at position 2. So, even in those cultivars of rape which are able to incorporate erucic acid at positions 1 and 3, where there is no (or at least reduced) discrimination against erucic acid, only a maximum 66% of the fatty acids incorporated into triacyl glycerols can be erucic acid. Such varieties of rape are known as HEAR (high erucic acid rape) varieties.

It would therefore be desirable to produce plants, eg conventional oil seed rape as well as HEAR varieties, which contain useful levels of trierucin and/or contain higher levels of erucic acid and/or contain oils with erucic acid incorporated at position 2; the same can be

said of oils of other vegetable oil crops such as maize, sunflower and soya, to name but a few examples. While in principle it may be thought possible to introduce into a desired plant DNA encoding a 2-acyltransferase of different fatty acid specificity, for example from a different plant, in practice there are a number of problems.

All enzymes involved in the acylation pathway for formation of triacylglycerols are membrane bound. These are the 1-acyltransferase, 2-acyltransferase and 3-acyltransferase which are present in the endoplasmic reticulum in the cytoplasm. They have not been purified. This makes working with them difficult and rules out the use of many conventional DNA cloning procedures. This difficulty does not, paradoxically, lie in the way of cloning the gene (or at least cDNA) encoding the Choroplastic 1-acyltransferase enzyme, which is soluble: in fact, recombinant DNA work has already been undertaken on this enzyme for a completely different purpose, namely the enhancement of chilling resistance in tobacco plant leaves, by Murata *et al* (*Nature* **356** 710-713 (1992)).

Wolter *et al*, Fat Science Technology, **93**, No 8: 288-89 (1991) suggested a strategy for cloning membrane bound enzymes such as 2-acyltransferases, although no exemplification was given.

WO-A-9413814 discloses a DNA sequence (and corresponding protein sequence) of a 2-acyltransferase. This sequence, which is derived from maize, is used to transform plants, such that the normal substrate specificity of the plants' 2-acyltransferase is altered. This disclosure also included the use of a cDNA sequence for a 2-AT derived

from maize to locate 2-ATs with a high degree of homology from both *Brassica* and *Limnanthes* species.

It has now been surprisingly found that there is in fact another 2-AT in *Limnanthes* which has no homologue in rape and which is seed specific. This 2-AT is able to incorporate erucic acid at the 2-position which the native 2-AT in rape, for example, is unable to do.

According to a first aspect of the invention, therefore, there is provided a recombinant or isolated DNA sequence, encoding an enzyme having membrane-bound 2-acyltransferase activity, and selected from:

(i) a DNA sequence comprising the DNA sequence of Figure 3 or its complementary strand,

(ii) nucleic acid sequences hybridising to the DNA sequence of Figure 3 or its complementary strand, under stringent conditions, and

(iii) nucleic acid sequences which would hybridise to the DNA sequence of Figure 3 or its complementary strand, but for the degeneracy of the genetic code.

Suitably, the DNA sequence of the invention comprises a DNA sequence as described in (i), (ii) or (iii) above which is the sequence of figure 3, or its complementary strand, or is one which has the characteristics of (ii) or (iii) where the sequence is the sequence of figure 3.

Fragments of the above DNA sequences, for example of at least 15, 20, 30, 40 or 60 nucleotides in length, are also within the scope of the invention.

Suitable stringent conditions include salt solutions of approximately 0.9 molar at temperatures of from 35°C to 65°C. More particularly, stringent hybridisation conditions include 6 x SSC, 5 x Denhardt's solution, 0.5% SDS, 0.5% tetrasodium pyrophosphate and 50μg/ml denatured herring sperm DNA; washing may be for 2 x 30 minutes at 65°C in 1 x SSC, 0.1% SDS and 1 x 30 minutes in 0.2 x SSC, 0.1% SDS at 65°C.

Recombinant DNA in accordance with the invention may be in the form of a vector, which may have sufficient regulatory sequences (such as a promoter) to direct gene expression. Vectors which are not expression vectors are useful for cloning purposes (as expression vectors themselves may be). Host cells (such as bacteria and plant cells) containing vectors in accordance with the invention themselves form part of the invention.

The 2-acyltransferase of the invention may be cloned directly, for example using complementation studies, from a DNA library of *Limnanthes*. For example, if *E. coli* is used as the complementation host, a mutant is chosen which is defective in the 2-acyltransferase; the DNA library from *Limnanthes* (e.g. *L. douglasii*) is transformed into the mutant complementation host; host cells containing the target acyltransferase gene can readily be selected using appropriate selective media and growth conditions. *E. coli* mutant JC201 is a suitable host for use in complementation studies relating to 2-acyltransferase.

Cloning the acyltransferase gene into a microbial host, such as a bacterium like *E. coli*, in such a way that the gene can be expressed has a particular advantage in that the substrate specificity of the acyltransferase gene can be assessed with membranes isolated from the microbial host before transformed plants are prepared, thereby saving considerably on research time. Such an assessment may be made by competitive substrate assays, in which differently detectably labelled candidate substrates for the enzyme compete with each other for incorporation into the glyceride. For example, ^{14}C-erucyl CoA and ^{3}H-oleoyl CoA can be used as competitive substrates for 2-acyltransferase, and the relative amounts of ^{14}C or tritium uptake into glyceride can be measured. (As 2-acyltransferases have acceptor, glycerol-based, substrates and donor, fatty acid-based, substrates, the experiment can be carried out with different acceptors, such as 1-erucyl-glycerol-3-phosphate and 1-oleoyl-glycerol-3-phosphate.) A gene coding for an enzyme which donates erucic acid to the acceptor (particularly 1-erucyl-glycerol-3-phosphate) may by this means be identified as a DNA sequence of choice for further use in the invention as described below.

Suitably, the DNA sequence of the invention encodes an enzyme having membrane-bound 2-acyltransferase activity.

The DNA sequence of the invention can be used to produce proteins which they encode, if desired. Thus, in a second aspect, the present invention provides an isolated protein which is the expression product of a DNA sequence of the invention. The protein may be expressed by host cells harbouring DNA in the form of an expression vector. The protein, an enzyme having 2-acyltransferase activity,

may have an amino acid sequence which is identical to or homologous with the sequence shown in Figure 3. The degree of homology will generally be greater than that of known proteins, and may be at least 40, 50, 60, 70, 80, 90, 95 or 99%. Suitably, the degree of homology will be 60% or greater, preferably 80% or greater and most preferably 90% or greater.

In a third aspect, the present invention provides an antibody capable of specifically binding to a protein of the invention.

In a fourth aspect of the invention, there is provided a plant having a 2-acyltransferase enzyme encoded by a DNA sequence as defined herein, wherein the enzyme is not a native enzyme of the plant.

While site-directed mutagenesis and/or other protein engineering techniques may be used to alter the specificity of an enzyme native to the plant, it is preferred that the plant be transgenic and incorporate an expressible 2-acyltransferase gene encoding the enzyme of the invention. For example, as described above, the 2-acyltransferase enzyme which does not discriminate against erucic acid, may be made by this means to express in a plant which would not normally incorporate erucic acid at the 2-position into triacylglycerides. An important embodiment of the invention relates to genetically engineered plants which contain trierucin. Such plants may thus also have higher levels of erucic acid incorporated into triacylglycerols than in corresponding non-engineered plants(eg. rape).

However, while a preferred approach is discussed above,

the invention includes modified 2-acyltransferase proteins obtained by methods well known in the art. The essential feature that such proteins should possess is, of course, the specificity for incorporating erucic acid at position 2 of TAGs. However, using a variety of techniques modified enzymes can be obtained which have, for example, greater heat stability, improved kinetic characteristics or even improved specificity for erucic acid.

Suitable examples of such engineered plants include *Brassica* eg *B.napus*, *B.campestris*, *B.Juncea* or *B.rapa*, maize, sunflower or soya.

For the 2-acyltransferase transgene to be expressible, a promoter has to be operatively coupled to it. Because at the present state of the art it is difficult precisely to regulate the site of incorporation of a transgene into the host genome, it is preferred that the transgene be coupled to its promoter prior to transformation of the plant. Promoters useful in the invention may be temporal- and/or seed-specific, but there is no need for them to be so: constitutive promoters may also be used provided they are suitably strongly expressed in the seed and are easier to isolate. Other tissues are unlikely to be adversely affected if the transgene encoding the acyltransferase enzyme is expressed in them, as the availability of the fatty acid CoA substrates is effectively limited to the seed.

The promoter-transgene construct, once prepared, is introduced into plant cells by any suitable means. The invention extends to such plant cells. Preferably, DNA is transformed into plant cells using a disarmed Ti-

plasmid vector and carried by *Agrobacterium* by procedures known in the art, for example as described in EP-A-0116718 and EP-A-0270822. Alternatively, the foreign DNA could be introduced directly into plant cells using an electrical discharge apparatus. This method is preferred where *Agrobacterium* is ineffective, for example where the recipient plant is monocotyledonous. Any other method that provides for the stable incorporation of the DNA within the nuclear DNA of any plant cell of any species would also be suitable. This includes species of plant which are not currently capable of genetic transformation.

The plants of the invention include ones which therefore have higher levels of erucic acid incorporated at the 2-position of their triacylglycerols (TAGs) as well as plants which contain trierucin.

Preferably DNA in accordance with the invention also contains a second chimeric gene (a "marker" gene) that enables a transformed plant or tissue culture containing the foreign DNA to be easily distinguished from other plants or tissue culture that do not contain the foreign DNA. Examples of such a marker gene include antibiotic resistance (Herrera-Estrella *et al*, *EMBO J.* **2**(6) 987-95 (1983) and Herrera-Estrella *et al*, *Nature* **303** 209-13 (1983)), herbicide resistance (EP-A-0242246) and glucuronidase (GUS) expression (EP-A-0344029). Expression of the marker gene is preferably controlled by a second promoter which allows expression in cells in culture, thus allowing selection of cells or tissue containing the marker at any stage of regeneration of the plant. The preferred second promoter is derived from the gene which encodes the 35S subunit of Cauliflower Mosaic

Virus (CaMV) coat protein. However any other suitable second promoter could be used.

In one embodiment of the invention, the transgenic plant's native 2-acyltransferase gene which corresponds to the transgene may be rendered at least partially inoperative or reduced in effectiveness by, for example, antisense or ribozyme techniques, as is known in the art.

A whole plant can be regenerated from a single transformed plant cell, and the invention therefore provides transgenic plants (or parts of them, such as propagating material) including DNA in accordance with the invention as described above. The regeneration can proceed by known methods.

Therefore, in a fifth aspect, the present invention provides a plant cell incorporating a DNA sequence of the invention.

In a sixth aspect, the invention provides seeds obtained from a plant of the invention.

By means of the invention, plants generating oil with a tailored lipid content may be produced. For example, plants which produce trierucin and/or incorporate erucic acid at position 2 of triacylglycerols (TAGs) can be engineered. In addition, the lipid composition of triacylglycerides in the plant may be substantially altered to produce triacylglycerides with a desired erucic acid content higher than has hitherto been possible. For example, oil seed rape (*B. napus*) may be transformed to produce oil whose triacylglyceride has an erucic acid content which is higher than that obtained in

untransformed plants. Similarly for other oil producing crops.

Promoters which naturally drive 2-acyltransferases may also be obtained by hybridisation and/or restriction enzyme analysis and/or sequencing studies using the Figure 3 sequence.

In further aspects, the present invention provides:

(a) a method of generating oil, the method comprising cultivating a plant of the invention and harvesting oil produced by the plant or a part (particularly seeds) thereof;

(b) oil obtained from a plant of the invention, or a part thereof, or from seeds of the invention which has erucic acid incorporated at the 2-position of its TAGs;

(c) oil obtained from a plant of the invention, or a part thereof, or from seeds of the invention which contains trierucin;

(d) a microbial host transformed with a DNA sequence of the invention;

(e) an oil seed rape plant, or other oil producing crop plant, containing trierucin;

(f) an oil seed rape plant, or other oil producing crop plant, having erucic acid incorporated at the 2-position of its TAGs; and

(g) a transgenic plant which expresses in at least some of its cells a DNA sequence of the invention. In particular, the DNA sequence is expressed in the seeds of the plant.

Preferred features of each aspect of the invention are as for each other aspect *mutatis mutandis*.

The invention is illustrated by the following examples. The examples refer to the accompanying drawings, in which:

FIGURE 1: shows the cDNA sequence derived in Example 2 and its derived protein sequence;

FIGURE 2: shows a comparison of the sequences of rape, maize and the *Limnanthes* '1' clone.

FIGURE 3: shows the cDNA sequence of pCB129 described in Example 3.

FIGURE 4: shows the results of a homology search using the sequence of Figure 3 against the OWL database;

FIGURES 5 and 6: show BESTFIT alignment of the sequence of Figure 3 compared with the sequence of *E. coli* 2-AT (Figure 5); as well as bestfit alignment of part of the *Limnanthes* sequence from Figure 3 with *E. coli* 2-AT (Figure 6). Lines indicate exact matches between the aligned sequences. Double points indicate conservative amino acid substitutions, and single points, pairs of amino acids conserved to a lesser degree;

FIGURE 7: shows an alignment of the sequence of Figure 3 with the top-matching sequence from *E. coli* 2-AT;

FIGURE 8: shows the results of Northern Blot analysis of RNA from *Limnanthes* embryo, leaf and stem probed with a fragment of the *Limnanthes* 2 (CB129);

FIGURE 9a-c: show the results of the Southern blot analysis described in Example 6.

FIGURE 10: shows the results of substrate specificity assays using membranes from JC201 (containing pCB129) and JC200;

FIGURE 11: shows the results of further substrate specificity assays using membranes from JC201 (containing pCB129) and JC200;

FIGURE 12: is a map of plasmid pSCV1.2 referred to in Example 8;

FIGURE 13: shows the results of the PCR procedure carried out in Example 9;

FIGURE 14: shows reverse-phase HPLC analysis of seed triacylglycerols; and

FIGURE 15: shows mass spectral assignment of trierucin.

EXAMPLE 1

Construction of library

Limnanthes douglasii plants were greenhouse-grown and seeds collected at stages III and IV, as defined for *Limnanthes alba* by Laurent and Huang, *Plant Physiol.*, **99**: 1711-1715 (1992) (majority were stage IV). Total RNA was isolated by a standard hot SDS method and mRNA purified by oligo dT-cellulose chromatography using methods detailed in the manual accompanying Pharmacia mRNA purification kit. A cDNA library was made from 5 µg polyA+ RNA using a cDNA synthesis kit from Pharmacia. The cDNA was primed with oligo dT and cloned as EcoRI fragments into the phage vector lambda ZAPII. A plasmid-based cDNA library was made from an unamplified aliquot of the lambda library by plasmid rescue with the helper phage R408 using protocols described in Delauney and Verma, *Plant Molecular Biology*, Manual **A14**: 1-23 (1990). During construction, 1 x 10^6 colonies (=cDNA clones) were plated and scraped into LB medium. This was grown for 3 hr and a plasmid preparation carried out to get a cDNA library representative of 1 x 10^6 cDNA clones from developing mid to late stage *Limnanthes* embryos.

EXAMPLE 2

Isolation of *Limnanthes* '1' clone homologous to maize clone

The *Limnanthes* cDNA '1' clone was obtained by heterologous screening of a seed cDNA library using a 600 bp *NcoI*/*PstI* fragment of the rape 2AT clone described in WO-A-9413814. This fragment corresponds to the N-terminus of the rape protein. Plaque hybridisation was in 6xSSC, 1xDenhardts, 0.5% sodium pyrophosphate and 1mM

EDTA (pre-hybridisation in the same solution minus EDTA and plus 50ug/ml denatured herring sperm DNA) and the filter was washed in 1xSSC at 60 C. The cDNA sequence of the hybridising clone (=pCB121) is shown in Figure 1. The relatedness between the rape, maize and *Limnanthes* '1' clones is shown in Figure 2.

EXAMPLE 3

Rescue of complementing cDNA clone - *Limnanthes* '2'

Complementation of the 2-AT mutant JC201 was done with the plasmid library as described in Brown *et al*, *Plant Mol. Biol.*, **26**: 211-223 (1994). 500 ng of DNA was used in the first transformation of the electroporation competent JC201, and after the second round of transformation with 50 ng of plasmid, substantially more colonies grew at 42°C with the cDNA plasmids than with plasmid vector alone (pBS SK⁺). 18 of these colonies were picked at random and cDNA clones isolated from each one. All 18 clones had the same size EcoRI insert of 1.1 kb and one, designated pCB129, was used in further studies.

Sequencing of pCB129

The restriction sites of the 1.1 kb insert in pCB129 were mapped. Both cut and re-ligated plasmids and smaller insert fragments subcloned into pBS SK⁺ were used as sequencing templates to obtain the sequence shown in Figure 3.

EXAMPLE 4

Homologies to other acyltransferases

The 281 amino acid open reading frame starting at the

first methionine was used as a probe sequence against the OWL database to search for homologous proteins. The top matches are shown in Figure 4. The ORF is much more homologous to the 2-AT PLSC of *E. coli* than the maize sequence of pMAT1, disclosed in WO-A-9413814. The *Limnanthes* protein is 27% identical to the *E. coli* protein PlsC, and if a smaller fragment of the protein is aligned, the identity is 38% over a 141 amino acid stretch (see Figure 6). An alignment with the top matching protein is in Figure 7.

EXAMPLE 5

Northern blot analysis

The open reading frame from pCB129 was labelled with ^{32}P and hybridised to a Northern Blot with 1 μg polyA+ RNA from *Limnanthes* embryo, leaf and stem at 42°C and the blot washed with 0.1 x SSC 0.1% SDS at 42°C. The gene is predominantly expressed in the embryo, with very low levels of expression in leaf and stem (see Figure 5).

EXAMPLE 6

Southern blot analysis

For the southern blots using plant DNAs, 2, 5 and 10ug samples of *Arabidopsis thaliana*, *Limnanthes douglasii* and *Brassica napus* DNA were cut with *Bam*HI, *Eco*RI and *Hind*III for separation and transfer. Hybridisations to all blots were done in the same hybridisation solution as described in example 2 above for the plaque hybridisation.

The *Limnanthes* '1' probe was a *Eco*RI/*Hind*III fragment of 1.3kb and the blot was washed in 2xSSC at 60 C to give the results shown in Figure 9a (higher stringency washes

using 0.2xSSC at 60 C with another blot still left 5-6 bands per rape lane). Two blots with *Limnanthes* '2' probes were also carried out. The first used the *Eco*RI insert of pCB129 as a probe and was washed in 2xSSC at 60 C to give the results shown in Figure 9b. The experiment was repeated with a probe corresponding to the ORF of pCB129, the result is shown in Figure 9c.

It is clear that homologues in rape exist for *Limnanthes* '1' at high stringency, but not for *Limnanthes* '2'.

EXAMPLE 7

Substrate specificity assays

JC201 containing pCB129 was grown in 200 ml culture and membrane fractions collected after sonication of the bacteria to cause lysis. The membrane pellet was collected at 200000 g after two clearing spins of 16000 g. The resuspended membranes were used in single substrate assays together with membranes from JC200 bacteria, which are wild-type for 2-AT.

The LPA acceptor in these assays was ^{32}P erucoyl LPA. This had been made from glycerol, [$\gamma^{32}P$]-ATP and erucoyl CoA using the enzymes glycerol kinase and over-produced 1-AT from arabidopsis (available in the lab). The LPA was purified from CoAs by thin layer chromatography on silica, extracted into methanol and resuspended in 0.2% octyl glucopyranoside after drying down.

For the assays, 100 μM LPA was used together with either 100 μM 18:1CoA or 100 μM 22:1CoA. Both JC200 and JC201 (pCB129) membranes were used separately in the assays. The experiment was repeated twice, with duplicate samples

taken the first time and single samples taken at more time points the second. The results are shown in Figures 10 and 11. Shorter incubation times were used in the second experiment to try and get linear incorporation with time for at least two sample points to get more accurate values for initial velocities.

Addition of pCB129 to JC201 enables the membranes to utilise 22:1 CoAs much more effectively than wild-type *E. coli* membranes from JC200. In the second experiment, the ratios of 18:1 to 22:1 CoA incorporation after 5 minutes are 1.45:1 for *Limnanthes* complemented membranes and 3.38:1 for JC200 membranes (see figures 10 and 11).

EXAMPLE 8

Construction of a plant expression vector of pCB129

The putative ORF of the cDNA sequence described in figure 3 was cloned into the plant expression vector pAR4 (napin) promoter and chalcone synthase (CHS) terminator cassette in Bluescript (Stratagene). It was necessary to use PCR to engineer an *Nco*I site at the putative start codon of the ORF. To avoid the need to PCR the full ORF and hence to reduce the possibility of introducing errors into the sequence, a 280 bp fragment was synthesised by PCR and cloned as an *Xba*I/*Bam*HI fragment into pCB130. pCB130 is a fully sequenced subclone of pCB129 with the 5' *Bam*HI fragment deleted. The resulting clone was named pCB141. The approx. 880 bp *Nco*I/*Sma*I fragment from pCB141 which encodes the putative ORF was excised and cloned into *Nco*I/*Sma*I sites of pAR4 resulting in pCB143. The *Xba*I/*Hind*III fragment of pCB143 comprising napin promoter -2-AT ORF CHS terminator was ligated with *Bgl*II linkers and cloned into plasmid SCV1.2 (figure 12), resulting in

SCV144. A second construct was developed by engineering an *Nco*I site approx. 100 bases downstream to allow translation to start at the second methionine of the *Limnanthes* 2 clone. The vector was contructed in the same manner as the SCV144.

EXAMPLE 9

Introduction via *Agrobacterium* into oilseed rape

The SCV-based vector SCV144 (referred to in Example 8) carrying the putative ORF under the control of a seed specific promoter were introduced into *Agrobacterium tumefaciens*. The resulting *Agrobacterium* strain was used to transform cotyledonary petioles of high erucic acid oilseed rape essentially as described in Moloney *et al* (*Plant Cell Reports*, **8**:238-242 (1989)). SCV144 carries the neomycin phosphotransferase (NPTII) gene allowing transformants to develop in the presence of the antibiotic kanamycin. Two transformation experiments (1000 cotyledons) were carried out.

Regenerant plants were grown to the four leaf stage and screened by polymerase chain reaction for the presence of the NPTII gene. The following primers were employed:

TN5 KAN1: 5' CGCAGGTTCTCCGGCGGCTTGGGTGG 3' (26 bases);
TN5 KAN2: 5' AGCAGCCAGTCCCTTCCCGCTTCAG 3' (25 bases).

The buffer employed was as follows:

10X = 100 mM TMS pH 8.8
500 mM KCl
15 mM $MgCl_2$
1% Triton X100

The following protocol was employed:

30 cycles of a) 20 secs at 97.5°C
b) 30 secs at 65°C
c) 90 secs at 74°C

1 cycle of 5 mins at 72°C and a slow decrease to room temp. The results are shown in Figure 13.

Fifty NPTII +ve plants were then grown to maturity and analysed by Southern blot for the presence of the sequence referred to in Example 3.

A microsome fraction was isolated from developing seeds. The tissue was homogenised with a polytron and the membrane fraction collected as a 200000 g pellet after a clearing spin of 40000 g. The membranes were washed with 0.5 M salt to remove extrinsic membrane proteins, and pelleted again at 200000 g before storage at -80°C.

The assays were carried out separately with 50 μM 18:1 LPA and 22:1 LPA and 100 μM 18:1CoA or 22:1CoA as acyl donors. The microsomes were capable of incorporating 22:1 CoA at position 2 of 1-acyl-glycerol-3-phosphate. Control microsomes from HEAR oilseed rape were unable to carry out this reaction.

Seed from 10 plants carrying the gene were analysed for the presence of trierucin molecules (see Taylor *et al*, *J. Am. Oil. Chem. Soc.*, **69**: 355-358 (1992) for analysis of trierucin content, and Christie, *Lipid Analysis, 2nd Edn., Pergamon Press, Toronto, Canada*: 158-161 (1982) for determination of amount of erucic acid at the 2-position) and for the level of erucic acid in the seed oil. A

range of levels of trierucin were evident (and the results are shown in table 1), whereas none is found in the untransformed line, and among the regenerants, some plants are found with levels of erucic acid over the level which is normally found in the HEAR untransformed line.

Table 1

Plant Number	% trierucin
1	0.03
2	2.8
3	0.4
4	2.9
5	2.7
6	0.01
7	2.1
8	1.0
9	0.1
10	1.4
control	0.0

EXAMPLE 10

TAG extraction:

Mature seeds were collected from transgenic plants. The seeds wre then extracted by homogenization with isopropanol (2ml) and then hexane (5ml). The extracts were filtered, the solvent was evaporated in a stream of nitrogen and the TAGs were taken up in acetone-acetonitrile (1:1, v/v; 1ml) containing BHT (50 mg/ml),and were stored at 4°C until analysed.

TAGs were analysed by reverse-phase high-performance liquid chromatography with a Gynkotek Model 480 pump and a Varex Model III evaporative light-scattering detector. The column consisted of two ChromSpher C18 (100 x 4.6mm; 3 micron particles) cartridge columns in series with guard column. The mobile phase was acetone-acetonitrile (1:1, v/v) and 10μl injected onto the column. With this system, trerucin eluted in approximately 17-20 minutes.

Gas chromatography:
Methyl esters of fatty acids were prepared by sodium methoxide-catalysed transesterification (Christie, W. W., *Gas Chromatography and Lipids*, Dundee; the oily press (1989)). They were analysed on a Hewlett Packard Model 5890 Series II gas chromatograph, fitted with split/splitless injection, and equipped with a capillary column (25m x 0.25mm x 0.2mm film thickness) of fused silica coated with CP-Wax 52CB. The carrier gas was hydrogen at a flow rate of 1ml/min. The initial temperature in the column was 170°C for 3 min, then the temperature was programmed to 210°C at 4°C/min, and held at this point for a further 25 min. Components were quantified by electronic integration.

Mass spectrometry:
The component co-chromatographing with trierucin was collected and pooled from five micropreparative HPLC runs under essentially the conditions described above. After removal of the solvent, the lipid in hexane solution was inserted via a syringe pump directly into the Finnigan SSQ 710C mass spectrometer with atmospheric pressure chemical ionization (APCI) at a corona voltage of 5.07 kV.

Pancreatic lipase hydolysis:
TAGs were subjected to pancreatic lipase hydrolysis by the method of Luddy *et al* (*J. Am. Oil Chem. Soc.*, **41**:693-696 (1964)). 1M Tris buffer (1 ml, pH 8), calcium chloride solution (0.1 ml; 2.2%) and bile salt solution (0.25ml; 0.05%) were added to the TAGs (5mg) and these were hydolysed with pancreatic lipase (pig pancreatin, Sigma) at 40°C for 2min. The reaction was stopped by the addition of ethanol (1ml) followed by 6M hydrochloric acid (1ml), and the solution extracted three times with diethyl ether (4 ml portions). The solvent layer was washed once with distilled water (3ml) and dried with sodium sulphate, before the solvent was removed on a rotary evaporator.

The required 2-monoacylglycerol products were isolated by micro-preparative HPLC on silica gel, ie. a column of Hypersil H3 (250 x 4.6mm). A Spectra-Physics Model 8700 solvent delivery system was used together with a Cunow Model DDL21 light-scattering detector (Severn Analytical). A stream-splitter (approximately 10:1) was inserted between the column and the detector to permit collection of fractions. the mobile phase was isohexane-methylterbutyl ether-acetic acid (100:100:0.02 by volume) at a flow rate of 1 ml/min. Monoacylglycerols eluted after about 14 min and were collected manually via the streamsplitter. They were methylated for GC analysis as before.

Results:
HPLC analysis of TAG
To initially identify plants which were expressing the *Limnanthes* LPA-AT protein, mature seed from transgenic plants was analysed for the presence ot trierucin. The

TAG fraction was extracted and examined by reverse-phase HPLC (figure 14).

Using this system trierucin was eluted at aprox. 17-20 mins; Figure 14A shows analysis of non-transformed *B.napus* in which no trierucin was detected. However, transgenic plants SCV144-2 and SCV144-9 were found to have 2.8% and 0.4%, repectively, of a lipid species which eluted at the same retention time as trierucin (Fig. 14B and C). A slight difference in the retention time for trierucin is apparent between the chromatograms illustrated in figure 14 as these were run on different occasions under slightly different conditions, but for each run the position of the trierucin peak was assigned by calibration with authentic trierucin.

The separation of the lipid species achieved by reverse-phase HPLC depends partly on the chain length and partly on the degree of unsaturation of the molecule.The elution time increases with the total number of carbon atoms in the fatty acid chain but is decreased by roughly the equivalent of 2C for each double bond. Therefore a TAG of C20:1-C22:1-C22:1 could elute in a similar but not identical place as trierucin (C22:1-C22:1-C22:1); trierucin would not be distinguished fromC20:1-C22:1-C24:1, if this TAG were also present. The results obtained strongly imply that erucic acid was being incorporated at *sn*-2 but for absolute confirmation of the identity of the trierucin peak mass spectrometry analysis was performed.

Mass spectral assignment of trierucin:
The components from seeds of SCV144-2 and SCV144-9 which co-chromatographed with trierucin were collected and

pooled from five micropreparative HPLC runs and subjected to mass spectral analysis. Figure 15A shows the spectrum of authentic trierucin and Fig. 15B that from transgenic seed of SCV144-9. The most abundant ion at m/z 1053.5 (labelled as 1053 and 1054 in (A) and (B) respectively) is the molecular ion [M+] for trierucoylglycerol. The ion at 715/716 represents loss of an erucate moiety. The results confirm the presence of trierucin and hence demonstrate that erucic acid was incorporated at *sn*-2 of TAG in the transgenic rape plants.

Fatty acid analysis of TAG:
The transgenic plants SCV144-2 and SCV144-9 were found to have only low levels of trierucin (2.8% and 0.4% respectively). Therefore to determine if the incorporation of erucic acid at the *sn*-2 position was limiting the production of trierucin, detailed positional analysis of fatty acids in TAG was performed. Total fatty acid composition was determined by gas chromatography (GC) of methyl esters. The identity of fatty acids at position 2 was determined by initial treatment of TAG with pancreatic lipase which removes the acyl groups from *sn*-1 and *sn*-3. The desired 2-monoacylglycerol products were isolated by micropreparative HPLC then methylated and examined by GC.

Under the growth conditions used the starting population had a maximum of 31.7 mol% erucic acid in the seed oil. Analysis of selected non-transformed rape plants showed that no erucic acid was incorporated at the 2 position. However, in SCV144-9 which had 0.4% trierucin and an erucic acid level of 32.2 mol%, erucic acid made up 9 mol% of the fatty acids esterified at *sn*-2. Similarly, in SCV144-2 which had 2.8% trierucin, erucic acid mahes up

32.1 mol% of total TAG fatty acids and 28.3 mol% of fatty acids esterified at *sn*-2. In these transgenic plants the amount of erucic acid at *sn*-2 appears to be correlated with the trierucin content.

CLAIMS

1. A recombinant or isolated DNA sequence selected from

(i) a DNA sequence comprising the DNA sequence of Figure 3 or its complementary strand;

(ii) nucleic acid sequences hybridising to the DNA sequence of Figure 3 or its complementary strand under stringent conditions; and

(iii) nucleic acid sequences which would hybridise to the DNA sequence of Figure 3, or its complementary strand, but for the degeneracy of the genetic code.

2. A DNA sequence as claimed in claim 1 which is a sequence having the characteristics of (i).

3. A DNA sequence as claimed in claim 1 or claim 2 which encodes an enzyme having membrane-bound 2-acyltransferase activity.

4. An isolated protein which is the expression product of a DNA sequence as defined in any one of claims 1 to 3.

5. A protein which is substantially homologous to a protein as claimed in claim 4.

6. An antibody capable of specifically binding to a protein as defined in claim 4 or claim 5.

7. A plant having a 2-acyltransferase enzyme encoded by a DNA sequence as defined in any one of claims 1 to 3,

wherein the enzyme is not a native enzyme of the plant.

8. A plant as claimed in claim 7, which is transgenic for the 2-acyltransferase enzyme.

9. A plant as claimed in claim 7 or claim 8, which is *Brassica*, maize, sunflower or soya.

10. A plant as claimed in claim 9 which is *B. napus*, *B. campestris*, *B. Juncea* or *B. rapa*.

11. A plant as claimed in any one of claims 7 to 10 which has higher levels of erucic acid incorporated into triacylglycerols.

12. A plant as claimed in any one of claims 7 to 11 which has erucic acid incorporated at the 2-position of its seed triacylglycerols (TAGs).

13. A plant as claimed in any one of claims 7 to 12 which contains trierucin.

14. A plant as claimed in any one of claims 7 to 13, wherein the transgenic 2-acyltransferase has a higher specificity for erucic acid than the native enzyme of the plant.

15. A plant as claimed in any one of claims 7 to 14, wherein the native 2-acyltransferase enzyme is at least partially rendered inoperative or removed, for example by a ribozyme or by antisense nucleic acid.

16. A plant cell incorporating DNA as defined in any one of claims 1 to 3.

17. A plant cell as claimed in claim 16 which is a cell of *Brassica*, maize, sunflower or soya.

18. A plant cell as claimed in claim 19 which is a cell of *B. napus*, *B. campestris*, *B. juncea* or *B. rapa*.

19. Seeds obtained from a plant as defined in any one of claims 7 to 15.

20. A method of generating oil, the method comprising cultivating a plant as claimed in any one of claims 7 to 15 and harvesting oil produced by the plant or a part (particularly seeds) thereof.

21. Oil obtained from a plant as defined in any one of claims 7 to 15, or a part thereof, or from seeds as defined in claim 19.

22. Oil as claimed in claim 21 which has erucic acid incorporated at the 2-position of at least a portion of its TAGs.

23. Oil as claimed in claim 21 which contains trierucin.

24. Oil as claimed in any one of claims 21 to 23 obtainable by a method as defined in claim 20.

25. A microbial host transformed with a DNA sequence as defined in any one of claims 1 to 3.

26. A fragment of a DNA sequence as claimed in any one of claims 1 to 3, comprising at least 15 nucleotides.

27. DNA encoding RNA which is antisense to sense RNA

encoded by DNA as claimed in any one of claims 1 to 3.

28. DNA encoding a ribozyme specific to RNA encoded by DNA as claimed in any one of claims 1 to 3.

29. Isolated or recombinant DNA containing a promoter which naturally drives expression of a gene to produce a protein as claimed in claim 5 or claim 6.

30. An oil seed rape plant, or other oil producing crop plant, containing trierucin.

31. An oil seed rape plant, or other oil producing crop plant, having erucic acid incorporated at the 2-position of its TAGs.

32. A transgenic plant which expresses in at least some of its cells a DNA sequence as defined in any one of claims 1 to 3.

33. A transgenic plant as claimed in claim 32 which is a *Brassica* plant.

34. A transgenic plant as claimed in claim 33 which is *B. napus*, *B. campestris*, *B. juncea* or *B. rapa*.

35. A transgenic plant as claimed in any one of claims 32 to 34 wherein the DNA sequence is expressed in the seeds of the plant.

36. A transgenic plant as claimed in any one of claims 33 to 35 which is a HEAR plant.

FIG. 1

FINAL LIM SEQ (CB121) - Genes

DNA sequence 1515 b.p. GAATTCGCGGCC ... GGCCGCGAATTC linear

```
   1 GAATTCGCGGCCGCCTACCGGCCATTCTAATTTTATATCCAAACGCCCCTCTCCATCTTCCTCGATTCATTTTTCTCGATC  80

  81 TTTCATCTCTTTTCCTACCATTCCTCTCTCTACAACATTCTTTTACACTATACATCCTTAGAGCTTCTCTTCCCTCATCGTT 160

 161 ATAGCCCCGAGCTAAAGCTGCC ATG GCG ATC CCT GCT GCA GCT TTC ATC GTA CCA ATA AGT CTT      223
       1                    M   A   I   P   A   A   F   I   V   P   I   S   L          14

 224 CTT TTT TTC ATG TCA GGC CTC GTT GTC AAT TTC ATT CAG GCA GTC TTC TAT GTT CTT GTT     283
  15  L   F   F   M   S   G   L   V   V   N   F   I   Q   A   V   F   Y   V   L   V      34

 284 CGG CCT ATT TCT AAG GAC ACA TAC AGA AGG ATC AAT ACG TTG GTG GCA GAA TTG TTG TGG     343
  35  R   P   I   S   K   D   T   Y   R   R   I   N   T   L   V   A   E   L   L   W      54

 344 CTA GAA CTT GTA TGG GTC ATT GAT TGG TGG GCA GGC GTT AAG GTC CAA TTA TAT ACT GAT     403
  55  L   E   L   V   W   V   I   D   W   W   A   G   V   K   V   Q   L   Y   T   D      74

 404 ACT GAG TCT TTC CGT CTA ATG CGT AAA GAA CAT GCA CTC TTA ATA TGC AAC CAC AGA AGT     463
  75  T   E   S   F   R   L   M   G   K   E   H   A   L   L   I   C   N   H   R   S      94

 464 GAC ATT GAC TGG CTC ATT GGA TGG GTC CTA GCA CAG CGA TGC GGC TGC CTC AGT TCT TCA     523
  95  D   I   D   W   L   I   G   W   V   L   A   Q   R   C   G   C   L   S   S   S     114

 524 ATA GCT GTT ATG AAG AAG TCA TCC AAA TTT CTC CCG GTA ATA GGT TGG TCT ATG TGG TTT     583
 115  I   A   V   M   K   K   S   S   K   F   L   P   V   I   G   W   S   M   W   F     134

 584 TCC GAA TAT CTC TTT CTC GAG AGG AAC TGG GCC AAA GAT GAA AAC ACT TTA AAG TCA GGT     643
 135  S   E   Y   L   F   L   K   R   N   W   A   K   D   E   N   T   L   K   S   G     154

 644 CTC CAG CGG CTG AAT GAC TTC CCT AAG CCT TTT TGG TTA GCT CTG TTT GTG GAA GGA ACT     703
 155  L   Q   R   L   N   D   F   P   K   P   F   W   L   A   L   F   V   E   G   T     174

 704 CGT TTC ACG AAA GCA AAA CTT CTA GCT GCT CAG GAA TAT GCA GCC TCT GCA GGA TTA CCC     763
 175  R   F   T   K   A   K   L   L   A   A   Q   E   Y   A   A   S   A   G   L   P     194
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764 GTG CCT CGA AAT GTT CTG ATT CCT CGT ACG AAG GGC TTT GTG TCA GCC GTT AGT AAC ATG  823
195 V   P   R   N   V   L   I   P   R   T   K   G   F   V   S   A   V   S   N   M  214

824 CGC TCA TTT GTC CCA GCT ATC TAT GAC TTG ACA GTC GCC ATT CCT AAA ACC ACC GAA CAA  883
215 R   S   F   V   P   A   I   Y   D   L   T   V   A   I   P   K   T   T   E   Q  234

884 CCT ACA ATG CTC AGA CTG TTC AGG GGG AAA TCT TCT GTG GTA CAC GTA CAC CTT AAG CGG  943
235 P   T   M   L   R   L   F   R   G   K   S   S   V   V   H   V   H   L   K   R  254

944 CAT TTG ATG AAG GAC TTC CCT AAA ACA GAT GAC GGT GTT GCA CAG TGG TGT AAA GAT CAA 1003
255 H   L   M   K   D   L   F   K   T   D   D   G   V   A   Q   W   C   K   D   Q  274

1004 TTT ATA TCC AAG GAT GCA TTG TTA GAC AAA CAT GTT GCT GAG GAT ACT TTC AGT GGC CTG 1063
275 F   I   S   K   D   A   L   L   D   K   H   V   A   E   D   T   F   S   G   L  294

1064 GAA GTG CAG GAC ATT GGT CGG CCA AGT AAG TCT CTT GTG GTG GTT GTC TCG TGG ATG TGC 1123
295 E   V   Q   D   I   G   R   P   M   K   S   L   V   V   V   V   S   W   M   C  314

1124 CTA CTC TGT TTG GGG CTT GTG AAA TTT CTT CAG TGG TCA GCA CTT TTA TCC TCA TGG AAG 1183
315 L   L   C   L   G   L   V   K   F   L   Q   W   S   A   L   L   S   S   W   K  334

1184 GGT ATG ATG ATA ACG ACA TTC GTT CTG GGA ATC GTG ACC GCC CTT ATG CAC ATG TTG ATA 1243
335 G   M   M   I   T   T   F   V   L   G   I   V   T   A   L   M   H   I   L   I  354

1244 CGT TCT TCC CAG TCA GAG CAT TCA ACC CCG GCA AAG ACG AGG GCC AGA CAA ACT GCA GAG 1303
355 R   S   S   Q   S   E   H   S   T   P   A   K   T   R   A   R   Q   T   A   E  374

1304 AAC CCA AAA TGA AATAAGCTTTTTTTCTTTATTAACGAACGGGTATATCAT ATG TAG TA ATG TGG GTT 1370
375 N   P   K   *                                           M   *     M   W   V    3

1371 TCC TTC ATT TAC CAA TGG ATT TAT GTT ATC AAT GCG GAA GAA TTA AGA TGT TTT TTT TTG 1430
  4 S   F   I   Y   Q   W   I   Y   V   I   N   A   E   E   L   R   C   F   F   L   L   23

1431 CCT TCC GGA GTT GTT TTA CTG TAT AGA CTT GTA TGC TGA AT ATG CAC AAT TAG AGAT ATG 1490
  24 P   S   G   V   V   L   L   Y   R   L   V   C   *       M   H   N   *       M    1

1491 TCA TTG TTT TAG CGGCCGCGAATTC                                                  1515
   2 S   L   F   *                                                                  5
```

FIG. 1 CONT'D

```
CLUSTAL V multiple sequence alignment

Lim 1 CB121   L   MAIPAAAFIVPISLLFFMSGLVVNFIQAVFYVLVRPISKDTYRRINTLVAELLWLELVWV
Maize         M   MAIPLVLVLPLGLLFLLSGLJVNAJQAVLFVTIRPFSKSFYRRINRFLAELLWLQLVWV
Rape          R   MAM-AAAVIVPLGILFFISGLVVNLLQ----------------------------------
                  **.     .*...**..***.** .*

              L   IDWWAGVKVOLYTDTESFRLMGKEHALLICNHRSDIDWLIGWVLAQRCGCLSSSIAVMKK
              M   VDWWAGVKVOLHADEETYRSMGKLHALIISNHRSDIDWLIGWILAQRSGCLGSTLAVMKK
              R   ----------------------------------RSGCLGSALAVMKK
                                                     * ***.*..*****

              L   SSKFLPVIGWSMWFSEYLFLERNWAKDENTLKSGLQRLNDFPKPFWLALFVEGTRFTKAK
              M   SSKFLPVIGWSMWFAEYLFLERSWAKDEKTLKWGLQRLKDFPRPFWLALFVEGTRFTPAK
              R   SSKFLPVIGWSMWFSEYLFLERNWAKDESTLKSGLQRLNDFPRPFWLALFVEGTRFTEAK
                  ********************.*******.*****  ***  ******.***.**********  **

              L   LLAAQEYAASAGLPVPRNVLIPRTKGFVSAVSNMRSFVPAIYDLTVAIPKTTEQPTMLRL
              M   LLAAQEYAASQGLPAPRNVLIPRTKGFVSAVSIMRDFVPAIYDTTVIVPKDSPQPTMLRI
              R   LKAAQEYAASSELPVPRNVLIPRTKGFVSAVSNMRSFVPAIYDMTVAIPKTSPPPTMLRL
                  * *********  **  ********************  ** ******* ** .**  .  *****.

              L   FRGKSSVVHVHLKRHLMKDLPKTDDGVAQWCKDQFISKDALLDKHVAEDTFSGLEVQDIG
              M   LKGQSSVIHVRMKRHAMSEMPKSDEDVSKWCKDIFVAKDALLDKHLATGTF-DEEIRPIG
              R   FKGQPSVVHVHIKCHSMKDLPESEDEIAQWCRDQFVTKDALLDKHIAADTFAGQKEQNIG
                  ..*..**.**..* * * ..* .... ....**.* *..*********.* .**  .     **

              L   RPMKSLVVVVSWMCLLCLGLVKFLQWSALLSSWKGMMITTFVLGIVTALMHILIRSSQSE
              M   RPVKSLLVTLFWSCLLLFGAIEFFKWTQLLSTWRGVAFTAAGMALVTGVMHVFIMFSQAE
              R   RPIKSLAVVLSWACLLTLGAMKFLHWSNLFSSWKGIALSALGLGIITLCMQILIRSSQSE
                  **.*** *  .  * *** .* . *. *. *.*.*.*. ...  ....*  *...*  **.*

              L   HSTPAK----------TRARQTAENPK
              M   RSSSARAARNRVK----------KEX
              R   RSTPAKVAPAKPKDNHQSGPSSQTEVEEKQK
                  .*..*.                .

                  * Conserved between 3 sequences
                  . Conserved between 2 sequences
```

FIG. 2

correct pCB129 cDNA -> 1-phase Translation

DNA sequence 1075 b.p. GTTCTATTCATG ... TCTTGAAAAAAA linear

```
1/1                                                 31/11
GTT CTA TTC ATG GCC AAA ACT AGA ACT AGC TCT CTC CGC AAC AGG AGA CAA CTA AAG CCG
V   L   F   M   A   K   T   R   T   S   S   L   R   N   R   R   Q   L   K   P
61/21                                               91/31
GCT GTA GCT GCT ACT GCT GAT GAT GAT AAA GAT GGG GTT TTT ATG GTA TTG CTA TCG TGT
A   V   A   A   T   A   D   D   D   K   D   G   V   F   M   V   L   L   S   C
121/41                                              151/51
TTC AAA ATT TTT GTT TGC TTT GCG GTA GTG TTG ATC ACG GCG GTG GCA TGG GGA CTA ATC
F   K   I   F   V   C   F   A   V   V   L   I   T   A   V   A   W   G   L   I
181/61                                              211/71
ATG GTC CTG CTC TTA CCT TGG CCT TAT ATG AGG ATT CGA CTA GGA AAT CTT TAC GGC CAT
M   V   L   L   L   P   W   P   Y   M   R   I   R   L   G   N   L   Y   G   H
241/81                                              271/91
ATC ATT GGT GGA TTA GTG ATA TGG ATT TAC GGA ATA CCA ATA AAG ATC CAA GGA TCC GAG
I   I   G   G   L   V   I   W   I   Y   G   I   P   I   K   I   Q   G   S   E
301/101                                             331/111
CAT ACA AAG AAG AGG GCC ATT TAT ATA AGC AAT CAT GCT TCT CCT ATC GAT GCT TTC TTT
H   T   K   K   R   A   I   Y   I   S   N   H   A   S   P   I   D   A   F   F
361/121                                             391/131
GTT ATG TGG TTG GCT CCC ATA GGC ACA GTT GGT GTT GCA AAG AAA GAG GTT ATA TGG TAT
V   M   W   L   A   P   I   G   T   V   G   V   A   K   K   E   V   I   W   Y
421/141                                             451/151
CCG CTA CTT GGA CAA CTA TAT ACA TTA GCC CAT CAT ATT CGT ATA GAT CGG TCA AAC CCG
P   L   L   G   Q   L   Y   T   L   A   H   H   I   R   I   D   R   S   N   P
481/161                                             511/171
GCT GCG GCT ATT CAG TCT ATG AAA GAG GCA GTT CGT GTA ATA ACC GAA AAG AAT CTC TCT
A   A   A   I   Q   S   M   K   E   A   V   R   V   I   T   E   K   N   L   S
541/181                                             571/191
CTG ATT ATG TTT CCA GAG GGA ACC AGG TCG GGA GAT GGG CGT TTA CTT CCT TTC AAG AAG
L   I   M   F   P   E   G   T   R   S   G   D   G   R   L   L   P   F   K   K
601/201                                             631/211
GGT TTT GTT CAT CTA GCA CTT CAG TCA CAC CTC CCG ATA GTT CCG ATG ATC CTT ACA GGT
G   F   V   H   L   A   L   Q   S   H   L   P   I   V   P   M   I   L   T   G
661/221                                             691/231
ACA CAT TTA GCA TGG AGG AAA GGT ACC TTC CGT GTC CGG CCA GTA CCC ATC ACT GTC AAG
T   H   L   A   W   R   K   G   T   F   R   V   R   P   V   P   I   T   V   K
721/241                                             751/251
TAC CTT CCT CCT ATA AAC ACT GAT GAT TGG ACT GTT GAC AAA ATC GAC GAT TAC GTC AAA
Y   L   P   P   I   N   T   D   D   W   T   V   D   K   I   D   D   Y   V   K
781/261                                             811/271
ATG ATA CAC GAC ATC TAT GTC CGC AAC CTA CCT GCG TCT CAA AAA CCA CTT GGT AGC ACA
M   I   H   D   I   Y   V   R   N   L   P   A   S   Q   K   P   L   G   S   T
841/281                                             871/291
AAT CGC TCA AAG TGA GTC GCT CTT TAC TCC AAG GTT AGC ATA ATG GAT ACG TAC TTT AGT
N   R   S   K   *   V   A   L   Y   S   K   V   S   I   M   D   T   Y   F   S
901/301                                             931/311
CTT GCT GCA TGA AAA GTT TAA TCC TTT CTT GTG ATA TTA GAT TAC AGC GTA AGA CTT TCA
L   A   A   *   K   V   *   S   F   L   V   I   L   D   Y   S   V   R   L   S
961/321                                             991/331
TGT TAA AGT AGT GTA ACA GTG CTT CTT GTT TGT AAC TTT TAC AAT AAA AGT ACC CTT TTG
C   *   S   S   V   T   V   L   L   V   C   N   F   Y   N   K   S   T   L   L
1021/341                                            1051/351
AAG AAG GGA GCA AGG TTT AAA TAG AAA CGA GTT CTA GTT CTT CTC TTG AAA AAA A
K   K   G   A   R   F   K   *   K   R   V   L   V   L   L   L   K   K
```

FIG. 3

FIG. 4

Best scores :

	Init	Opt	%Id	O'lap		
1	142	279	35.3	187	PLSC_YEAST	POSSIBLE 1-ACYL-SN-GLYCEROL-3-PHOSPHATE ACYLTRANSFERASE (EC 2.3.1.51). - SACCHAROMYCES CEREVISIAE (BAKER'S YEAST).
2	227	279	27.9	244	PLSC_ECOLI	1-ACYL-SN-GLYCEROL-3-PHOSPHATE ACYLTRANSFERASE (EC 2.3.1.51). - ESCHERICHIA COLI.
3	225	273	27.5	244	PLSC_SALTY	1-ACYL-SN-GLYCEROL-3-PHOSPHATE ACYLTRANSFERASE (EC 2.3.1.51). - SALMONELLA TYPHIMURIUM.
4	225	247	32.1	187	B45582	ParF=small hydrophobic protein - Salmonella typhimurium
5	61	122	22.1	195	AAS_ECOLI	2-ACYLGLYCEROPHOSPHOETHANOLAMINE ACYLTRANSFERASE/ACYL-ACYL CARRIER PROTEIN SYNTHETASE. - ESCHERICHIA COLI.
6	56	113	28.6	56	VE5_HPV33	PROBABLE E5 PROTEIN. - HUMAN PAPILLOMAVIRUS TYPE 33.
7	62	100	25.3	87	NU5M_DROYA	NADH-UBIQUINONE OXIDOREDUCTASE CHAIN 5 (EC 1.6.5.3). - DROSOPHILA YAKUBA (FRUIT FLY).
8	80	100	28.6	56	VE5_HPV58	PROBABLE E5 PROTEIN. - HUMAN PAPILLOMAVIRUS TYPE 58.
9	61	96	20.9	134	YSCH817913	YSCH8179 NCBI gi: 488176 - Saccharomyces cerevisiae
10	53	96	17.1	205	A45264	rBAT=77.8 kda protein related to b0,+ amino acid transporter system for dibasic and neutral amino acids - rabbit
11	53	96	17.1	205	A45048	rBAT-2=amino acid transport protein - rabbit
12	56	94	18.5	108	HSMALB	HSMALB NCBI gi: 435480 - Homo sapiens
13	55	93	16.0	156	JS0734	endo-1,4-beta-xylanase (EC 3.2.1.8) precursor - fungus (Filobasidium floriforme)
14	80	93	19.7	71	DESA_SYNY3	FATTY ACID DESATURASE (EC 1.14.99.-). - SYNECHOCYSTIS SP. (STRAIN PCC 6803).
15	54	91	18.0	122	HPCFPA	HPCFPA NCBI gi: 469211 - Hepatitis C virus
16	82	91	21.1	71	SYCDESA	SYCDESA NCBI gi: 488509 - Synechocystis sp.
17	76	90	26.9	78	QAY_NEUCR	QUINATE TRANSPORTER. - NEUROSPORA CRASSA.
18	65	86	27.3	66	OUTO_ERWCH	TYPE 4 PREPILIN-LIKE PROTEIN SPECIFIC LEADER PEPTIDASE (EC 3.4.99.-) (PECTIC ENZYMES SECRETION PROTEIN OUTO). - ERWINIA CHRYSANTHEMI.
19	61	85	34.1	44	YORR_TTV1	HYPOTHETICAL 7.4 KD PROTEIN. - THERMOPROTEUS TENAX VIRUS 1 (STRAIN KRA1) (TTV1).
20	75	85	19.2	52	SYODESA2	SYODESA2 NCBI gi: 488511 - Synechococcus sp.
21	72	84	31.0	42	YIJE_ECOLI	HYPOTHETICAL 34.1 KD PROTEIN IN KATG-GLDA INTERGENIC REGION (O312). - ESCHERICHIA COLI.
22	56	84	20.0	75	MAL_HUMAN	T-LYMPHOCYTE MATURATION-ASSOCIATED PROTEIN. - HOMO SAPIENS (HUMAN).
23	69	83	22.5	80	JQ1724	E1 membrane glycoprotein precursor - canine coronavirus (strain Insavc-1)
24	53	83	29.4	51	YGL1_YEAST	HYPOTHETICAL MEMBRANE PROTEIN IN LEU1-SCL1 INTERGENIC REGION. - SACCHAROMYCES CEREVISIAE (BAKER'S YEAST).

BESTFIT alignment of E. coli 2AT (PLSC) - top line and Limnanthes pCB129 protein - bottom line.

```
                Gap Weight:  3.000      Average Match:  0.540
              Length Weight:  0.100   Average Mismatch: -0.396

                    Quality:  138.7             Length:    250
                      Ratio:  0.573              Gaps:      4
      Percent Similarity: 50.207    Percent Identity: 26.971

      c.a x k.a                    November 14, 1994  10:05  ..

                                .         .         .         .         .
E coli            1 MLYIFRLIITVIYSILV...CVFGSIYCLFSPRNPKHVATFGHMFGRLAP 47
                    |:.:  : | |.:.:::   ..:| | .|: |:  .::  :|:::|:: .
Lim 2 CB129      32 MVLLSCFKIFVCFAVVLITAVAWGLIMVLLLPWPYMRI.RLGNLYGHIIG 80
                                .         .         .         .         .
                 48 ...LFGLKVECRKPTDAESYGNAIYIANHQNNYDMVTASNIVQPPTVTVG 94
                      :: . :..: ......  .||||.||.. .| . .  :.. .||.|:
                 81 GLVIWIYGIPIKIQGSEHTKKRAIYISNHASPIDAFFVMWLAPIGTVGVA 130
                                .         .         .         .         .
                 95 KKSLLWIPFFGQLYWLTGNLLIDRNNRTKAHGTIAEVVNHFKKRRISIWM 144
                    ||.::|.|::|||| |. :: |||.|.. | ..: |.|. :.. .:|: |
                131 KKEVIWYPLLGQLYTLAHHIRIDRSNPAAAIQSMKEAVRVITEENLSLIM 180
                                .         .         .         .         .
                145 FPEGTRSRGRGLLPFKTGAFHAAIAAGVPIIPVCVSTT..SNKINLNRLH 192
                    ||||||| :  |||||.| .| |:.. :||:|:.:..|  . : .  |::
                181 FPEGTRSGDGRLLPFKKGFVHLALQSHLPIVPMILTGTHLAWRKGTFRVR 230
                                .         .         .         .         .
                193 NGLVIVEMLPPIDVSQYGKDQVRELAAHCRSIMEQKIAELDKEVAEREAA 242
                     . :.|. ||||:..::. |.: :..  .:.|  ..::. :|.::. : .
                231 PVPITVKYLPPINTDDWTVDKIDDYVKMIHDIYVRNLPASQKPLGSTNRS 280
```

FIG. 5

FIG. 6

BESTFIT alignment of part of Limnanthes pCB129 protein with E. coli 2-AT (PLSC)

```
                Gap Weight:  3.000      Average Match:  0.540
              Length Weight:  0.100   Average Mismatch: -0.396

                    Quality:   97.3             Length:     141
                      Ratio:  0.700              Gaps:        1
          Percent Similarity: 54.676   Percent Identity: 38.129

          c.a x kdl.a                December 1, 1994  11:39  ..

                                .         .         .         .         .
E coli     67 AIYIANHQNNYDMVTASNIVQPPTVTVGKKSLLWIPFFGQLYWLTGNLLI 116
              ||||.||.. .| . .  :.. .||.|:||.::|.|::|||| |. :: |
Lim         2 AIYISNHASPIDAFFVMWLAPIGTVGVAKKEVIWYPLLGQLYTLAHHIRI  51
                                .         .         .         .         .
           117 DRNNRTKAHGTIAEVVNHFKKRRISIWMFPEGTRSRGRGLLPFKTGAFHA 166
               ||.|.. | ..: |.|. :.. .:|: |||||||| :  |||||.| .|
            52 DRSNPAAAIQSMKEAVRVITEENLSLIMFPEGTRSGDGRLLPFKKGFVHL 101
                                .         .         .         .
           167 AIAAGVPIIPVCVSTT..SNKINLNRLHNGLVIVEMLPPID 205
               |:.. :||:|:.:..|  . : .  |:: . :.|. ||||:
           102 ALQSHLPIVPMILTGTHLAWRKGTFRVRPVPITVKYLPPIN 142
```

Limnanthes (pCB129)=K versus E. coli 2-AT (P1sC)=C

CLUSTAL V multiple sequence alignment

```
coli
Lim 2 CB129

C   MLY----------IFRLIITVIYSILVC----------VFGSIYC
K   MAKTRTSSLRNRRQLKPAVAATADDDKDGVF-MVLLSCFKIFVCFAVVLITAVAWGLIMV
                .*  ...   . *.**          * *

C   LFSPRNPKHV---ATFGHMFGRLAP-LFGLKVECRKPTDAESYGNAIYIANHQNNYDMVT
K   LLLPWPYMRTRLGNLYGHIIGGLVIWIYGIPIKIQGSEHTKK--RAIYISNHASPIDAFF
    *. *.   ..    .**..* *   ..*. . . . ..      ****.** .  *

C   ASNIVQPPTVTVGKKSLLWIPFFGQLYWLTGNLLIDRNNRTKAHGTIAEVVNHFKKRIS
K   VMWLAPIGTVGVAKKEVIWYPLLGQLYTLAHHIRIDRSNPAAAIQSMKEAVRVITEENLS
        ** *.**  ..* *..**** *. .. ***.* . *  .. * *  .  .*

C   IWMFPEGTRSRGRGLLPFKTGAFHAAIAAGVPIIPVCVSTT--SNKINLNRLHNGLVIVE
K   LTMFPEGTRSGDGRLLPFKKGFVHLALQSHLPIVPMILTGTHLAWRKGTFRVRPVPITVK
    . ********** .  ***** *  * *. . .**.*. ... *  . .  *..  . *

C   MLPPIDVSQYGKDQVRELAAHCRSIMEQKIAELDKEVAEREAAGKV
K   YLPPINTDDWTVDKIDDYVKMIHDIYVRNLPASQKPLGSTNRSK--
    ****.  .  *... .  *  ....  .* ..  . .
```

FIG. 7

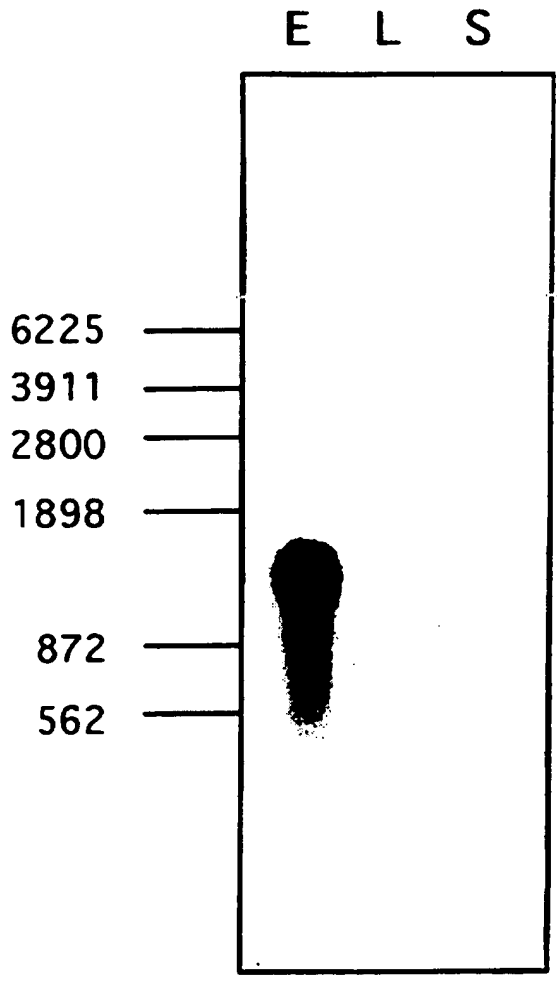


FIG. 8.

FIG. 9A

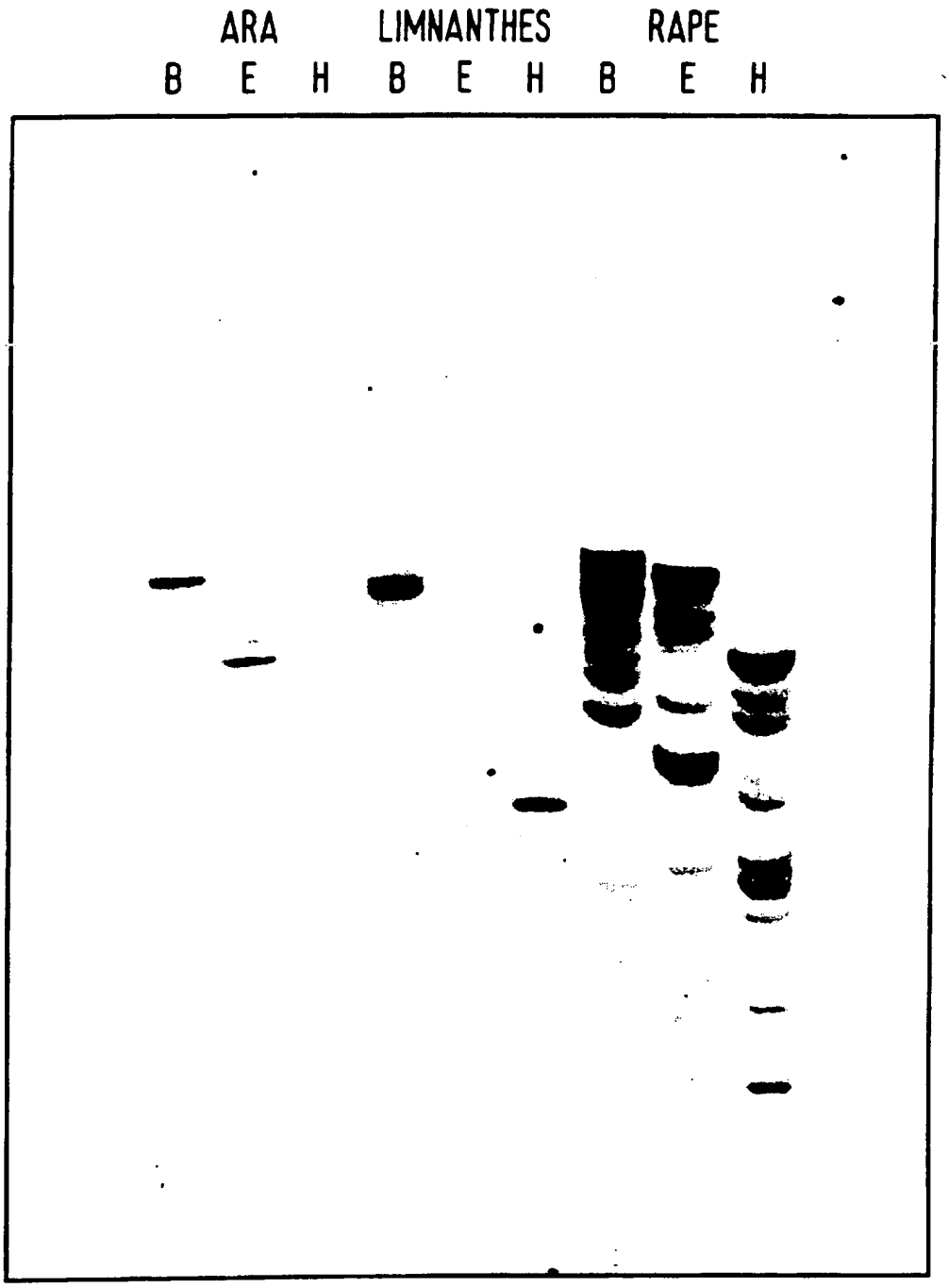


FIG. 9B

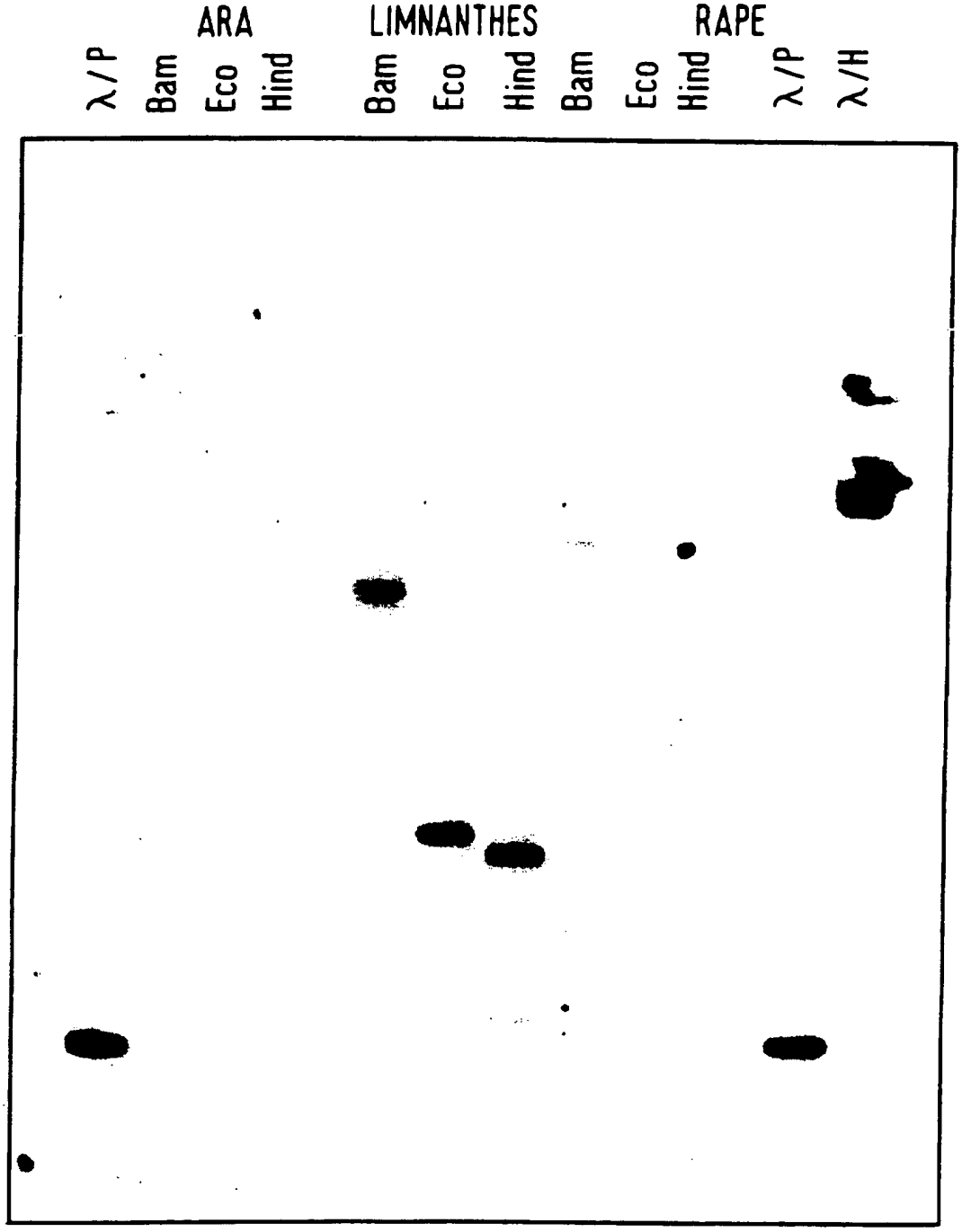

ARA
LIMNANTHES
RAPE
λ/P
Bam
Eco
Hind
Bam
Eco
Hind
Bam
Eco
Hind
λ/P
λ/H

FIG. 9C

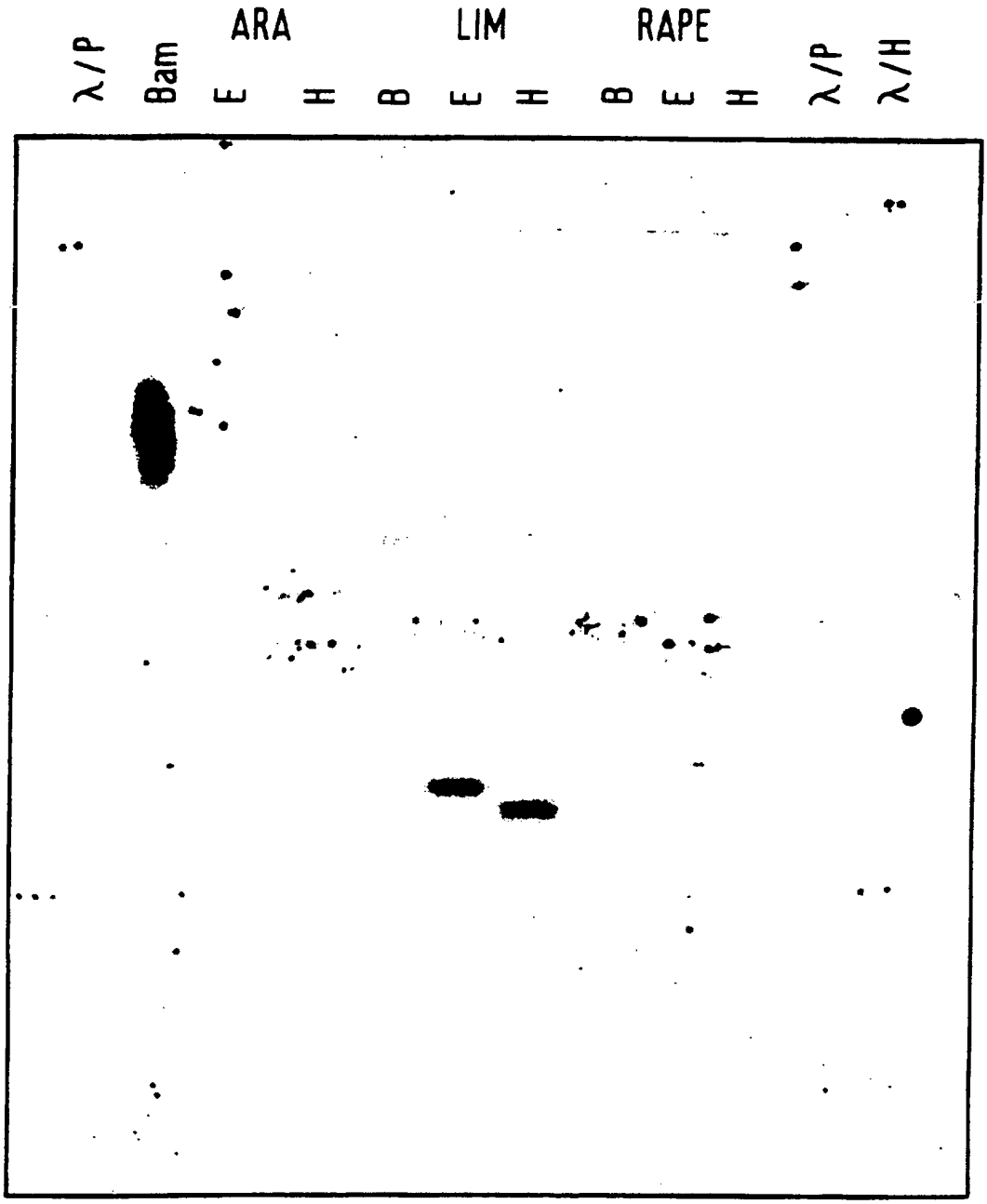


FIG. 10

JC200 AND JC201 (pCB129) WITH 100 MICROMOLAR 22:1 LPA
AND 100 MICROMOLAR 18:1 OR 22:1 CoA

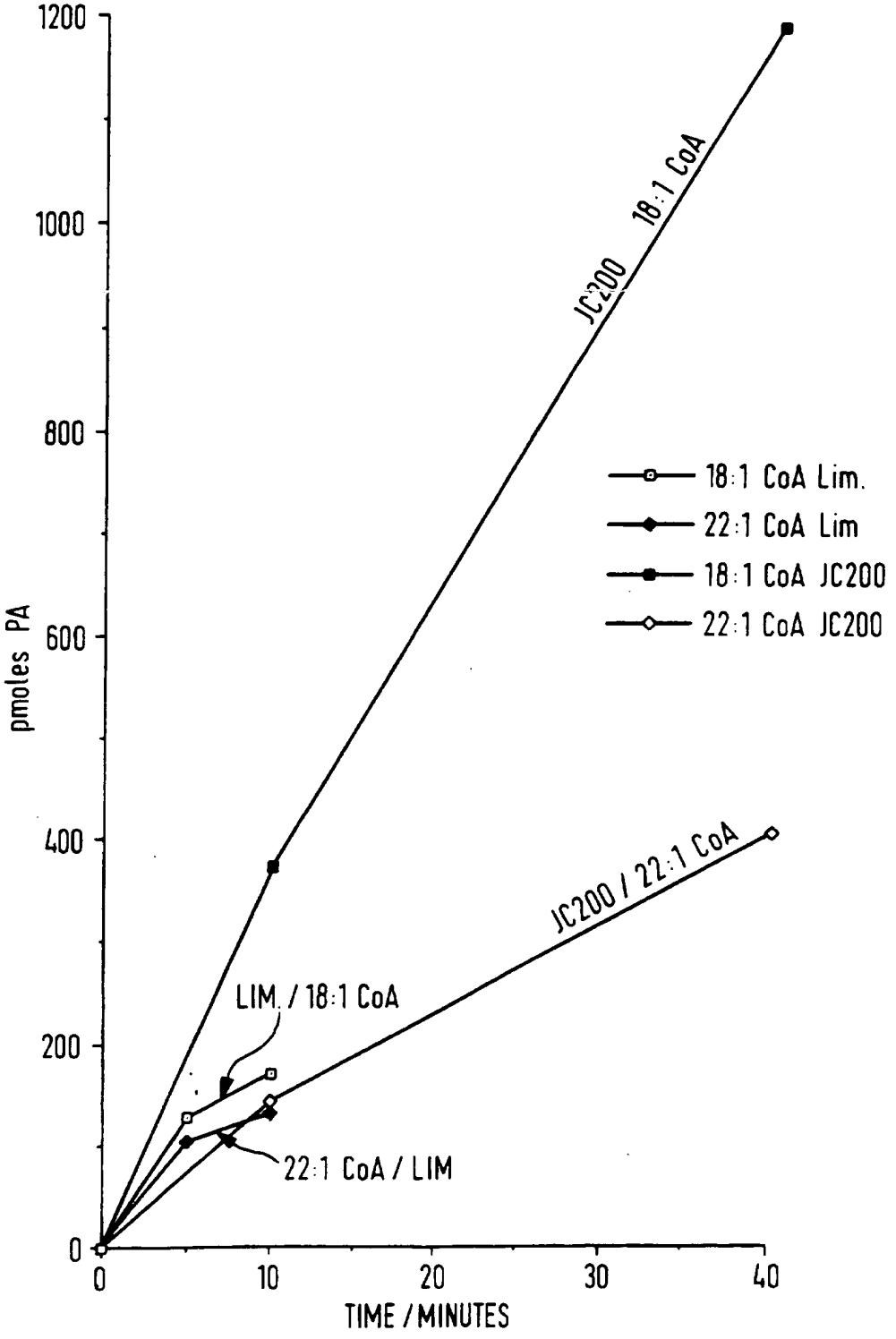
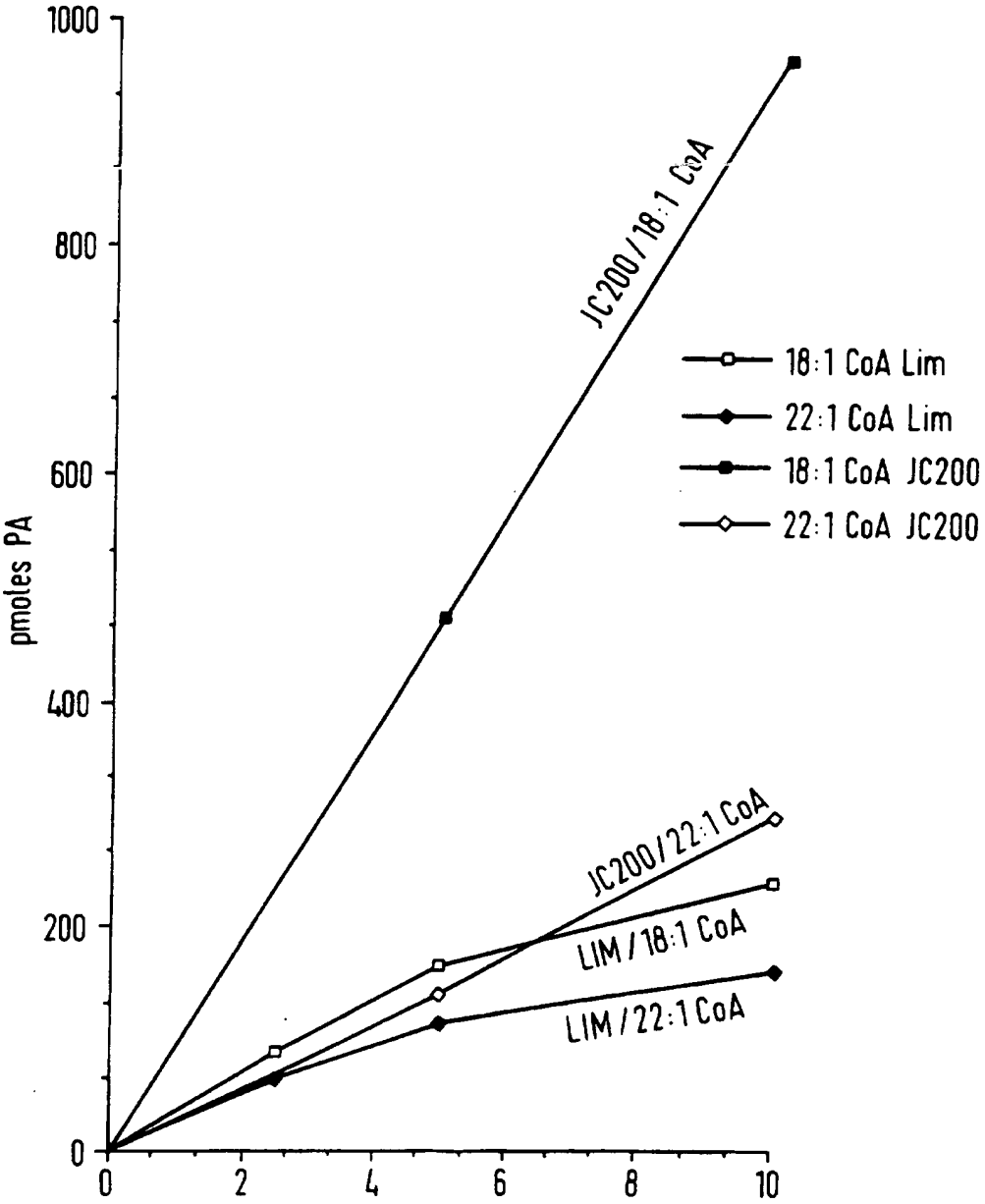


FIG. 11

JC200 AND JC201 (pCB129) WITH 100 MICROMOLAR 22:1 LPA AND 100 MICROMOLAR 18:1 OR 22:1 CoA 2nd EXPERIMENT



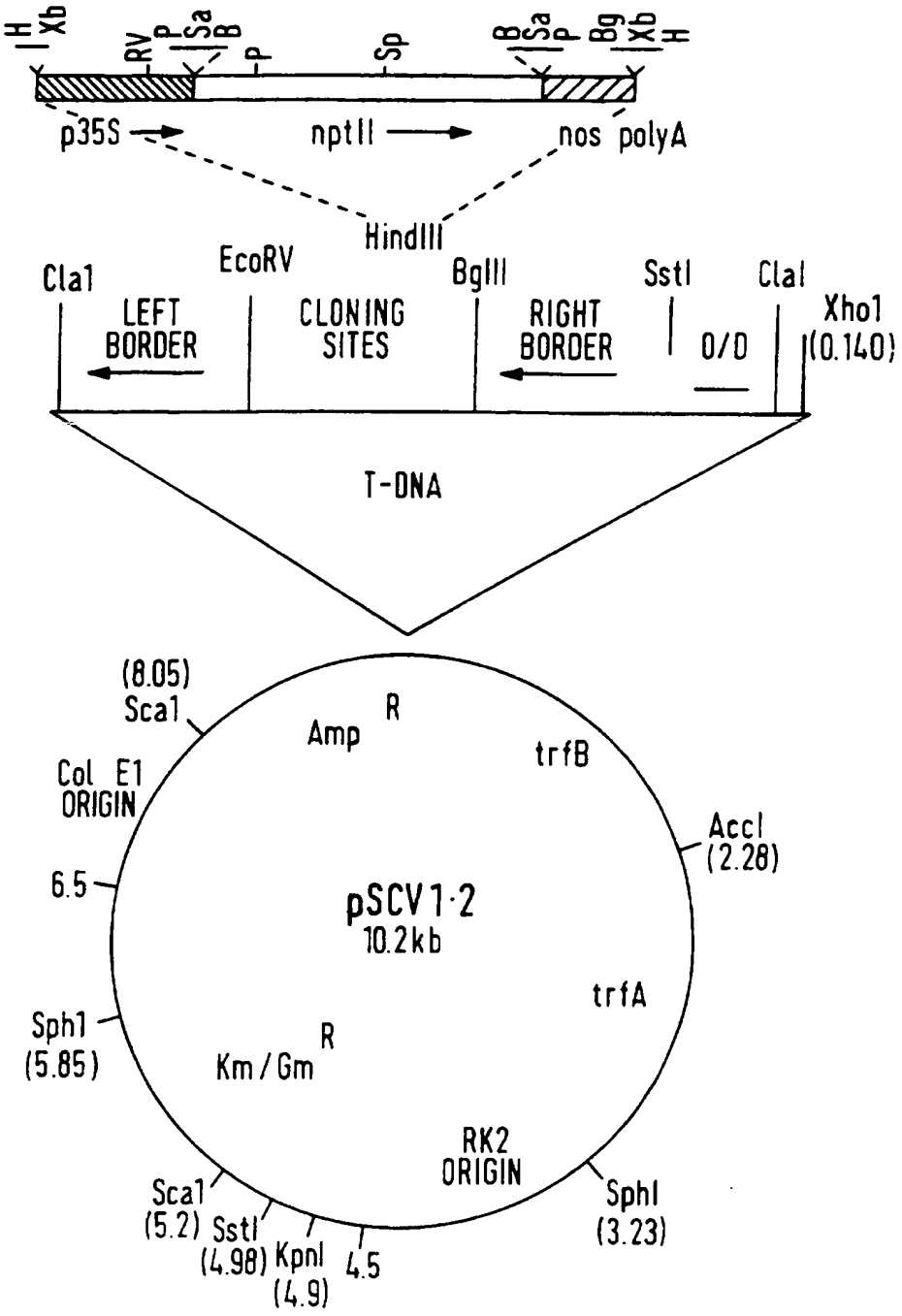

H Xb RV P Sa B P Sp B Sa P Bg Xb H
p35S
nptII
nos polyA
HindIII
ClaI
EcoRV
BglII
SstI
ClaI
XhoI
(0.140)
LEFT BORDER
CLONING SITES
RIGHT BORDER
O/D
T-DNA
(8.05)
ScaI
Amp
R
trfB
Col E1 ORIGIN
AccI
(2.28)
6.5
pSCV1·2
10.2kb
trfA
SphI
(5.85)
R
Km/Gm
RK2 ORIGIN
ScaI
(5.2)
SstI
(4.98)
KpnI
(4.9)
4.5
SphI
(3.23)

FIG. 12

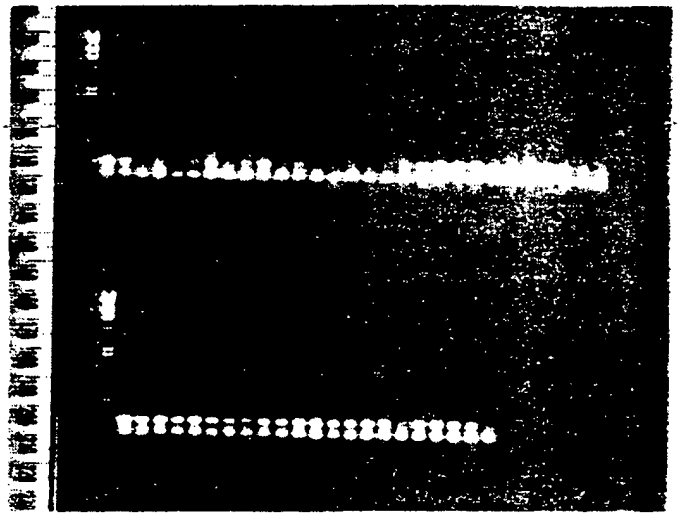


FIG. 13

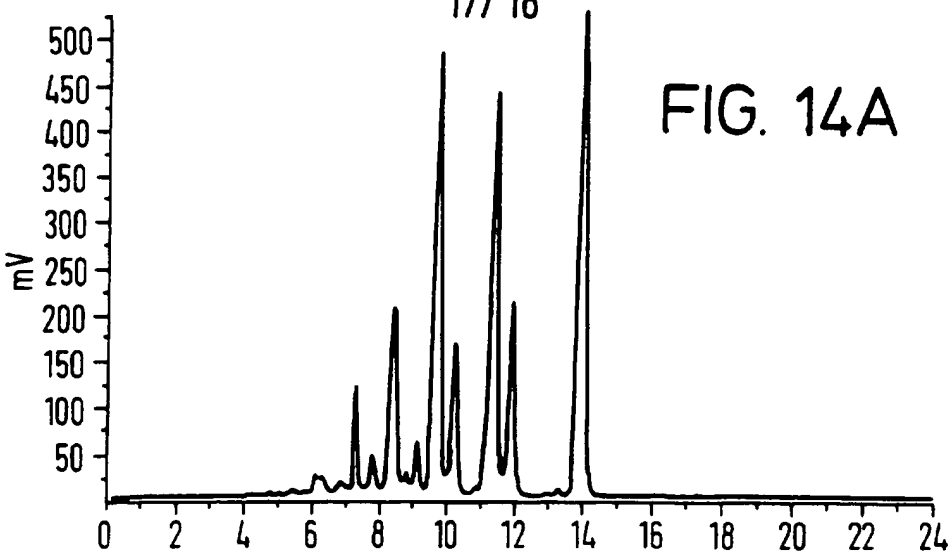


FIG. 14A

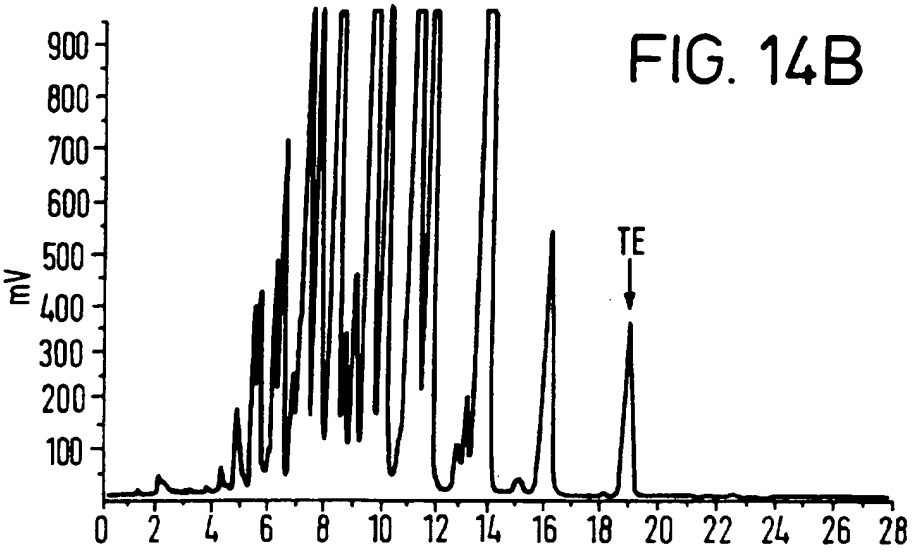


FIG. 14B

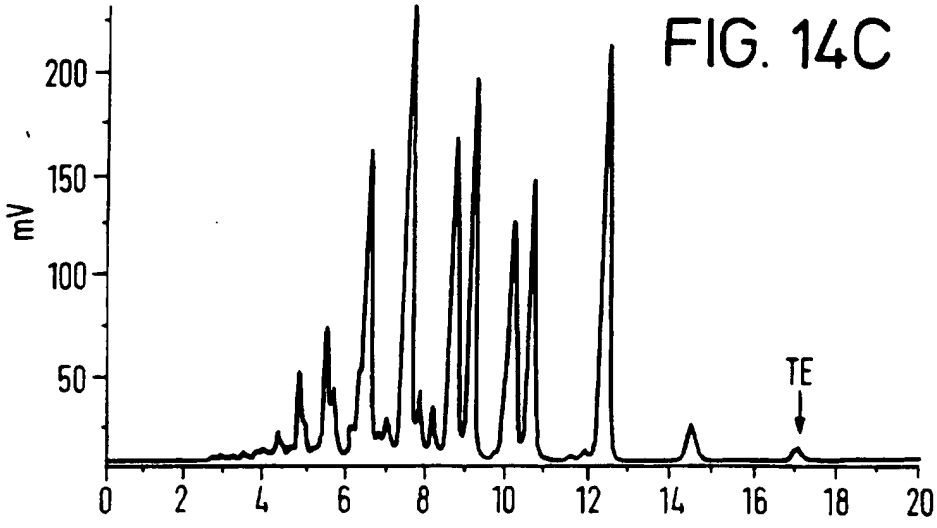


FIG. 14C

FIG. 15A

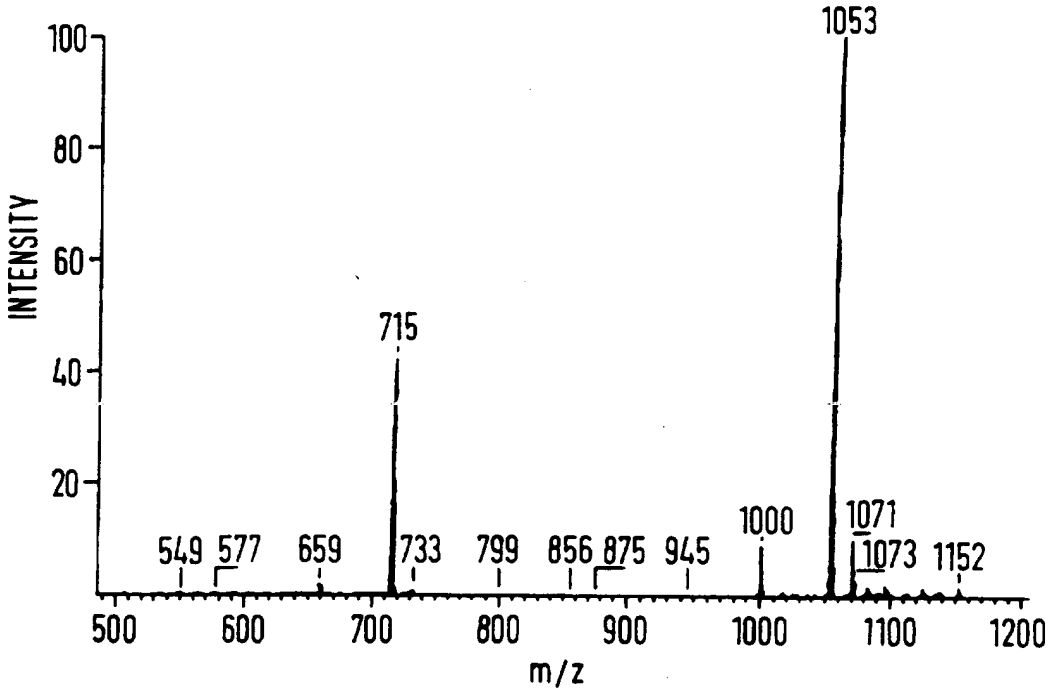
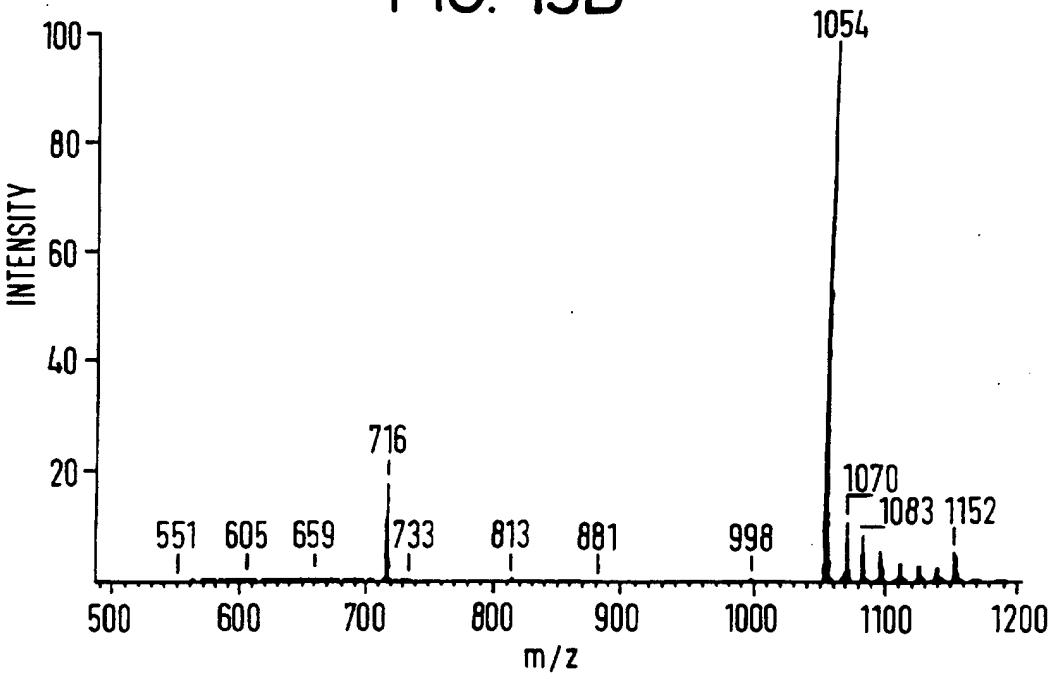


FIG. 15B



INTERNATIONAL SEARCH REPORT

Ir ational Application No
PCT/GB 96/00306

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/54 C12N15/82 A01H5/00 C11B1/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N A01H C11B

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	PLANT LIPID METAB., [PAP. INT. MEET. PLANT LIPIDS], 11TH (1995),INTERNATIONAL MEETING ON PLANT LIPIDS, PARIS, FRANCE, JUNE 26-JULY 1,1994. 531-3. EDITOR(S): KADER, JEAN-CLAUDE; MAZLIAK, PAUL. PUBLISHER: KLUWER, DORDRECHT, NETH., XP002000898 HANKE, C., ET AL.: "cDNA clones from Limnanthes douglasii encoding an erucoyl-CoA specific 1-acylglycerol-3-phosphate acyltransferase"	1-5,25, 26
Y	see the whole document	6-24, 27-36

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

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"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

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"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

1

Date of the actual completion of the international search	Date of mailing of the international search report
22 May 1996	17.06.96

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Maddox, A

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) .DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	CHEMICAL ABSTRACTS, vol. 119, no. 19, 1993 Columbus, Ohio, US; abstract no. 199618, TAYLOR, D.C., ET AL.: "Biosynthesis of triacyl glycerols in Brassica napus L. cv Reston; Target: trierucin" XP002000907 see abstract & SEED OILS FUTURE. EDS: MACKENZIE, S.L., ET AL., AOCS, CHAMPAIGN, ILL., 1992, pages 77-102, ---	6
Y	FAT SCIENCE TECHNOLOGY, vol. 93, no. 8, 1991, pages 288-290, XP002000899 WOLTER, F.P., ET AL.: "Biochemical and molecular biological approaches for changing the fatty acid composition of rape seed oil" see the whole document ---	7-24, 27-36
Y	WO,A,94 13814 (NICKERSON BIOCEM LTD ;SLABAS ANTONI RYSZARD (GB); BROWN ADRIAN PAU) 23 June 1994 see the whole document ---	15,27-29
X	BIOLOGICAL ABSTRACTS, vol. 94, 1992 Philadelphia, PA, US; abstract no. 136764, LOEHDEN, I., ET AL.: "Triacylglycerol biosynthesis in developing seeds of Tropaeolum majus L. and Limnanthes douglassi R. Br." XP002000906 see abstract & PLANTA, vol. 188, no. 2, 1992, pages 215-224, ---	4,5
X	JOURNAL OF THE AMERICAN OIL CHEMISTS SOCIETY, vol. 69, no. 4, 1992, pages 355-358, XP002000900 TAYLOR, D.C., ET AL.: "Formation of trierucoylglycerol (Trierucin) from 1,2-dierucoylglycerol by a homogenate of microspore derived embryos of Brassica napus L." see the whole document ---	21-24

1

-/--

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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X	JOURNAL OF THE AMERICAN OIL CHEMISTS SOCIETY, vol. 71, no. 2, February 1994, pages 163-167, XP002000901 TAYLOR, D.C., ET AL.: "Stereospecific analysis of seed triacylglycerols from high-erucic acid Brassicaaceae: Detection of erucic acid at the sn-2 position in Brassica oleracea L. genotypes"	21-24, 30,31
Y	see page 166, right-hand column, last paragraph	36
P,X	PLANT PHYSIOLOGY (ROCKVILLE) 109 (4). 1389-1394., November 1995, XP002003604 LASSNER M W, ET AL.: "Lysophosphatidic acid acyltransferase from meadowfoam mediates insertion of erucic acid at the sn-2 position of triacylglycerol in transgenic rapeseed oil." see the whole document	1-3, 7-14, 16-24, 26,27, 30-36
P,X	PLANT MOL. BIOL. 29(2), 267-78, October 1995, XP002000905 BROWN A P: "Identification of a cDNA that encodes a 1-acyl-sn-glycerol-3-phosphate acyltransferase from Limnanthes douglasii." see the whole document	1-3
P,X	WO,A,95 27791 (CALGENE INC ;DAVIES HUW MAELOR (US); HAWKINS DEBORAH (US); NELSEN) 19 October 1995 see the whole document	1-3
A	JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 268, no. 29, 15 October 1993, pages 22156-22163, XP002000902 NAGIEC, M.M., ET AL.: "A suppressor gene that enables Saccharomyces cerevisiae to grow without making sphingolipids encodes a protein that resembles an Escherichia coli fatty acyltransferase" see the whole document	1-3

-/--

INTERNATIONAL SEARCH REPORT

Ir tional Application No
PCT/GB 96/00306

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	PLANTA, vol. 185, 1991, pages 124-131, XP002000903 HARES, W., ET AL.: "Substrate specificities of the membrane-bound and partially purified microsomal acyl-CoA:1-acylglycerol-3-phosphate acyltransferase from etiolated shoots of Pisum sativum (L.)" see the whole document	4,5
A	MOLECULAR AND GENERAL GENETICS, vol. 232, 1992, pages 295-303, XP002000904 COLEMAN, J., ET AL.: "Characterization of the Escherichia coli gene for 1-acyl-sn-glycerol-3-phosphate acyltransferase (plsC)" see the whole document	4,5
E	WO,A,96 09394 (NORDDEUTSCHE PFLANZENZUCHT HAN ;SAATVEREDELUNG LIPPSTADT BREME (DE) 28 March 1996 see the whole document	1-3
L	EUROPEAN JOURNAL OF BIOCHEMISTRY, vol. 232, no. 3, 15 September 1995, pages 806-810, XP000560132 HANKE C ET AL: "A PLANT ACYLTRANSFERASE INVOLVED IN TRIACYGLYCEROL BIOSYNTHESIS COMPLEMENTS AN ESCHERICHIA COLI SN-1-ACYLGLYCEROL-3-PHOSPHATE ACYLTRANSFERASE MUTANT" This document has been cited to confirm the sequence of the gene cloned by Hanke et al., disclosed at the 11th international meeting on plant lipids	1-3

1

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/GB 96/00306

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-9413814	23-06-94	AU-B-	5656794	04-07-94
		CA-A-	2151147	23-06-94
		EP-A-	0673424	27-09-95
		HU-A-	71785	28-02-96
		PL-A-	309327	02-10-95
		SK-A-	76395	13-09-95
WO-A-9527791	19-10-95	NONE		
WO-A-9609394	28-03-96	DE-A-	4433307	21-03-96